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DR. R. GREIG-SMITH IN THE CHAIR.

THE FIXATION OF NITROGEN BY THE NODULE-FORMER.

BY R. GREIG-SMITH, D.SC.

During the past few years, a renewed interest has been awakened in the application of bacterial cultures of the nodule-former to leguminous crops, chiefly by the work of Moore, who endeavoured to show that the media employed for the growth and conveyance of the bacteria had been too nutritious, and the micro-organisms had in consequence become enfeebled. By accustoming them to a poor medium and keeping them there, he claimed that they remained more vigorous and more capable of fixing atmospheric nitrogen. The reasoning appears to be sound, and the results of the American experiments, as recorded by Moore, favour the idea. I believe, however, that European tests have not been so decisive, while Australian experiments have been negative.

It may be said—for one does not know in detail the methods adopted by the various manufacturers of "nitragin"—that cultures of bacteria for application to a particular crop have been grown from a micro-organism originally isolated from a nodule on the root of a plant of the same crop, and furthermore, that it has been assumed that all the bacteria in the nodules have the same activity. It is true that experiment may justify this assumption, but by the time that the test has been completed, the microbe may have lost its potency. If, however, we doubt the assumption, we realise that we know very little about the microbe, and that until we know more, we cannot hope to advance very far in being able to explain its variability or to utilise it advantageously in agriculture. In considering the properties of the microbe, we are met by the possibility that it may not be capable of fixing nitrogen, *per se*, that it may act, as it were,

by suggestion, providing a something which enables the cells of the nodule or of the plant to do the fixation. Some colour was lent to this idea by the fact that Beijerinck and a number of other investigators, including myself, could not prove a fixation in artificial culture. On the other hand, Mazé (1898) showed that a fixation did occur, and since that time it has been confirmed by Moore and by Löhnis (both in 1905). I have recently been able to corroborate the results of these workers, and to indicate under what conditions the fixation occurs. My experiments have brought out certain points in the physiology of the microbe that ought to be considered in selecting a bacterium for the preparation of trade cultures. The researches have been contributed in detail to the Proceedings of the Linnean Society of New South Wales for 1906, but as they may not be readily accessible to all who are interested in the subject, I take this opportunity of bringing before a wider circle of readers the results of my work, and the conclusions to which I have been led regarding the functions and activities of the microbe.

The microscopical examination of sections of the root-nodules on the majority of leguminous plants shows, stretching from cell to cell, structures known as "infection-threads." These have been shown to consist of slime, and to contain imbedded in them the nodule bacteria. After a time the slime disappears, and the microbes appear free. If these bacteria are transferred to an artificial saccharine medium, they, as a rule, produce slime readily. From the fact that slime is formed immediately after the isolation of the bacteria, we can reason that it is being continually formed by the living microbes within the nodules. Even the absence of an infection-thread in the tissues of the lupin nodules does not indicate that the bacteria are incapable of forming these structures, for I have isolated lupin bacteria and have seen the threads produced by them in acidified media. After a time the threads lost their sharp outlines, and the bacteria appeared as felted chains of the irregular forms, known as bacteroids. Since the essential constituent of the slime is made gelatinous by acids, it is probable that the presence of the slime as an infection-thread in the young nodule is made visible by the acid of the nodular sap, and its disappearance is occasioned by the secretion of a substance by the microbes which slowly softens the toughened slime. Certain races of the bacterium undoubtedly secrete

enzymes, which digest the slime after its formation in artificial culture.

But the main point to be observed is that the bacteria can and do form slime within the nodule, and as it is not exuded, it must be utilised. We have, therefore, in the slime a product of the bacterium, which is in all probability advantageous to the plant.

The great majority of the bacteria in the nodule are dead (about 99·8 per cent.), but still there are probably enough living cells (about one and a quarter millions in a nodule one-eighth of an inch in diameter) to maintain an active formation of products. As the dead bacteria colour deeply with the ordinary aniline stains, we know that they are not in process of solution, and therefore that the actively growing plant cannot be benefited by the intra-bacterial albuminoids, as some writers would lead us to believe. The living bacteria, when transferred to artificial media, exhibit differences in their slime-forming capacities. Some do not produce it upon any kind of artificial medium, others do not do so immediately, but may be induced to form it by repeated and rapid transference upon suitable pabula. The majority form more or less slime upon all kinds of saccharine media, but a few, while producing it upon artificial media with a plant basis (*e.g.*, saccharose-bean-agar), refuse to do so upon an entirely synthetic medium. Thus the slime-forming function is variable. It is furthermore variable in its persistence. An optimum formation can only be maintained with the majority of the races of the microbe by keeping up a rapid growth (transfers made at least twice a week). The minority do not appear to be quite so sensitive in this respect. The most sensitive are those which readily secrete slime-digesting enzymes.

Some of my best slime-forming races were examined to see if a fixation of nitrogen had taken place, and I found that a gain had occurred. The fixation is so small, however, that it is necessary to continue the experiments for three weeks or a month, at the end of which time the amount gained, after making due allowance for original nitrogen, &c., by check tests, is equivalent to from 0·15 to 0·6 c.c. of tenth normal alkali per 20 c.c. test, that is to say, from 1 to 4 mgrms. of nitrogen per 100 c.c. of medium. In my results, I have always expressed as zero an equivalent of less than 1 mgrm. The gain is small, but it was repeatedly obtained, and there can be no

doubt that the bacterium is capable of fixing atmospheric nitrogen under the same conditions that ensure the formation of slime. Under conditions which hinder or prevent slime-formation, such as an unsuitable carbohydrate, there is little or no fixation even when there is a proliferation of cells. Conversely, conditions which favour the production of slime, such as the presence of another bacteria (*e.g.*, *Bac. levaniformans*, which can neither fix nitrogen nor form slime from dextrose, the sugar generally employed), also increase the fixation. The individual tests did not show that the gain was proportional to the formation of slime, but this could probably be accounted for by the comparatively large experimental error, the variation in the gum-content of the slimes, and a more or less autodigestion. By collecting the results, however, and taking averages, the relation between these two functions became evident. This is shown in the following table :—

Nitrogen gained, mgrms. ..	0·0	1·0	2·0	3·0	4·0
Slime formed, grms.	2·6	5·7	7·1	8·6	16·3
Number of tests included ..	16	14	16	7	3

I have a recollection that some writer advanced the idea that fixation could only occur in the acid tissues of the nodule. Such is not the case, for my experiments showed that fixation took place when the bacteria were on a medium originally and permanently alkaline Indeed, from my work I see no reason why this micro-organism should not be credited with the power of being able to gather nitrogen from the air during its sojourn in the soil. A source of carbon is necessary, but this is also required by other soil bacteria, *e.g.*, *Azotobacter chroococcum*, that are credited with this important function. In artificial media, *Azotobacter* and *Rhizobium leguminosarum*, the nodule-former, make a powerful combination.

The importance of the slime is now apparent, and the consideration of its properties might tell us something more about its uses, while the investigation of the physiological activity of the microbe would probably tell us much concerning the formation of slime and, inferentially, of the fixation of nitrogen.

With regard to the nature of the slime, I have shown that, like all bacterial slimes, the essential constituent is a gum, which may be obtained from it by a method already

described (this J., 1904, 105). It is soluble in water and becomes gelatinous upon treatment with acids. As a rule, the solutions are more or less gelatinous, but this may have been partly or entirely caused by the acid in the faintly acid slimes during the preparation of the gum. The chemical reactions of gums prepared from slimes that were produced by several races of the bacterium were much the same, while the decomposition products were identical.*

There is a bacterium, *Vibrio denitrificans, Sewerin*, which, while it is morphologically the same as the nodule-former, acts in an opposite manner and, as its name implies, converts the combined nitrogen of nitrates into nitrogen gas. It also produces slime on saccharine media, and the gum obtained therefrom is identical with that from the nodule-former. From this we infer that the formation of the irregular bacteroidal forms has no relation with the fixation of nitrogen as some writers suppose and that their formation is a function of the chemical nature of the gum, a condensation product of which constitutes the capsule of the bacterium. The bacteroids, like the infection-threads, probably result from the action of the acid of the sap, or that formed by the microbes in artificial media, toughening the capsule and preventing the ready separation of the intracapsular contents.

The sugars obtained from the gum by hydrolysis are the same as those derived from the carbohydrate of the nucleoproteid molecule of *Dematium pullulans*, and possibly of the nucleoproteids of higher plants. According to Kossel, the carbohydrate of certain nucleoproteids is hydrolysed to glucose and a pentose. The absence of a definite name for the pentose raises the suspicion that the gum in question had been incompletely investigated.

* A strong solution (mucilage) of the gum is coagulated by alcohol, basic and ammoniacal lead acetates, ferric chloride, phosphotungstic acid, and by copper sulphate, followed by potassium hydrate. Neutral lead acetate or barium hydrate produces a clot, a precipitate, or a gelatinisation. Tannic acid gives an opalescence, and no reaction is obtained with copper sulphate, Fehling's solution, ammonio-copper hydrate, milk of lime, silver nitrate or iodine.

The gum is optically active, and may be either dextro- or lævo-rotatory; that from three races of the bacterium had the specific rotations $[\alpha]_D = -31{\cdot}75°, +29{\cdot}7°$ and $+31{\cdot}7°$. It is easily hydrolysed by 5 per cent. sulphuric acid to a mixture of dextrose and galactose, the former of which preponderates. Furfural is given off during hydrolysis.

and, indeed, it is possible that the production of furfural had been taken as indicating the pentose. Since gums which give galactose as the only recognisable sugar also yield furfural, this test for the pentoses is of no value by itself. Indeed, all bacterio-vegetable gums and possibly all other gums contain a greater or less amount of those indefinite, reducing substances which have been named furfuroïds. There is therefore reason to believe that the gum of *Rhizobium* is peculiarly adapted for building up the nucleoproteid molecule.

As in the case of all the bacterial slimes that I have examined this slime is nitrogenous, and even after being purified by repeated precipitation from aqueous solution by alcohol the gum still contains nitrogenous material. In one case, for example, 0·73 per cent. of nitrogen was found in the dry ash-free gum. Since the bacterium can fix atmospheric nitrogen, and since the slime is nitrogenous while the dead bacteria of the nodule are not in process of solution during the active growth of the plant, it is evident that it is by means of the slime that the host-plant is benefited, and that probably both nitrogenous matter and carbohydrate serve to build up the nucleoproteids of the higher plant.

The formation of nodules by the bacterium need not be considered as the result of an irritating parasitic action of the micro-organism as some writers imagine, but rather as the consequence of the production of nutrients at that place, the cells in the immediate vicinity of the microbe being better nourished and therefore growing faster than others at a distance. The bacterium is no parasite; there is a true symbiosis between it and the plant. The latter supplies the saline and carbohydrate nutrients, while the former elaborates a nitrogenous slime which is utilised by the plant.

The bacteria are not restricted to the nodule, but may be found at other places. I have isolated them from the stems of lupins, but when taken from these relatively very acid places, they are incapable of forming slime, although by appropriate treatment they can be induced to regain some of the power which they had lost.

The production of slime, which can be measured with the balance, enabled the physiological activities of several races of the bacterium to be investigated with some degree of precision. I use the term "bacterium" in a general sense, for, thanks to having been able to examine the

microbe while producing slime luxuriantly (when it was, so to speak, " plump "), I have been able to show that it is a compound organism, allied to *Leuconostoc* or the *Streptococcus* and consisting of cocci contained within a rod-shaped or branching capsule. On this account, I still prefer to name it by Frank's designation, *Rhizobium leguminosarum*, rather than by Beijerinck's *Bacillus radicicola* or *Pseudomonas radicicola* of some American workers.

For the formation of slime a source of carbon is essential, and it may be either dextrose, lævulose, saccharose, maltose, or mannit. Glycerin will also serve, but lactose is a very poor nutrient. The use of whey, therefore, as a basis for the preparation of a medium would probably lead to disappointment. Although there is no necessity for supplying a source of nitrogen, still for a luxuriant and rapid production of slime it is advisable to add some nitrogenous substance; asparagin and nitrate are best, peptone and urea are good, while ammonium salts, such as the phosphate and sulphate, are bad unless organic salts are present.

The majority of the races showed individual differences in response to the action of single nutrients, which showed that they were physiologically different. It was curious that three races from Kräl, of Prague, *viz.*, lupin, pea, and bean, occupied a group by themselves; there was very little difference between them, and they differed greatly from my Australian races. This may have been on account of their having lost their original slime-forming power and having been induced to regain it under laboratory conditions. This is quite in keeping with my work upon other gum bacteria, the conclusions from which have led me to believe, and, indeed, one infection experiment clearly brought it out, that the host-plant can bring about an alteration in the gum-forming faculty of a bacterium lodging in its tissues, and cause it to produce a particular kind of gum. The majority of the bacteria which are responsible for the production of the vegetable gums belong to a group of microbes, and when this is considered in relation with the action of the host-plant, we have the possibility that there is, or was, one original race or type.

It is believed that the bacteria from the nodules of one species of plant are identical, and this was partly borne out in my experiments. Races taken from the nodules

of field peas in March and October were physiologically similar. The same could be said about races taken from the nodules of a single plant, *Robinia pseudacacia*. But the pea race from Král was absolutely different from the Australian pea races, and I have obtained many races from the blue lupin. One of these grew well upon vegetable media, but not at all upon synthetic media, and I have obtained other three distinct races from a *single* nodule. There is, therefore, no rule in the matter, and a bacterium taken from a nodule may or may not be identical with its companions.

The optimum temperature for the production of slime is about 22° C., but a Robinia race was exceptional in having an optimum of 26° C. A neutral or slightly alkaline medium is most favourable, but slime can still be produced upon a medium containing originally 0·033 per cent. of phosphoric acid, and probably, finally, about 0·066 per cent. of acid calculated as phosphoric. In one case, a Robinia race, the slime-formation was still active with 0·05 per cent. of phosphoric acid in the original medium. An exception to the rule that an alkaline medium is best was found in a race isolated from the blue lupin. It was acidophile, inasmuch as it formed very little slime upon slightly alkaline media, while a luxuriant formation was obtained upon media originally neutral, but soon becoming acid.

Since the power of fixing atmospheric nitrogen is bound up with the formation of slime, there is little wonder that there have been failures with the trade cultures, for although sugars, such as saccharose and maltose, and other carbonaceous substances, such as mannit and glycerin, have been used in the culture media, the formation of slime as a symptom of the potency of the bacterium has not hitherto been demonstrated, and consequently no special means have been adopted for maintaining this function. Furthermore, since we have had no means of determining the nitrogen-fixing power, the media used for propagating the microbes may have been faulty, as, for example, the whey-gelatin employed by Continental workers and firms. Mazé is apparently the only writer who has noted that there was some relation between fixation and slime, his observations having led him to the conclusion that as fixation was always accompanied by the formation of slime, the latter was the substance which conveyed the nitrogen to the host-plant.

The bacterium produces the most slime on media the nitrogenous and saline matter of which approximate to what is found in soil water, and I am, therefore, inclined to think that media containing the equivalent of about 0·06 per cent. of asparagin and 0·1 to 0·2 per cent. of alkaline phosphate is better adapted for maintaining the potency of the bacteria than the comparatively nitrogen-free media of Moore.

We have seen that the bacteria from the various species of leguminous plants differ physiologically, but not to a greater extent than the microbes from a single nodule may do, and as it is extremely probable that the host-plant can sooner or later modify the activities of the microbe to its own requirements, it is likely that one single race will suit all leguminous plants. This is quite in agreement with the experiments of Moore, who found that any one organism could infect nearly all leguminous plants, provided that it had been grown for some time upon synthetic nitrogen-free media. But it is also evident from the work that I have done, that the microbe must also be capable of producing slime. If it cannot, or if it can do it but feebly, the time required for the regeneration of the power within the tissues of the root-hairs may be too long for the microbe to be of any considerable use to the plant.

There is much to recommend the employment of a culture common for all crops, but the great difficulty would be to know what would be the best race to select. The discovery of a microbe which is acidophile, while the majority are basophile, raises the question whether or not it would be better to employ such a race. As the bacterium has to work in the acid tissues of the root-hairs and of the nodule, we should expect an acidophile race to be superior to all others, inasmuch as it would be less affected by the varying acidity of the nodular sap, with which we must connect the death of the vast majority of the bacteria. Failing the use of such an acidophile race, and, perhaps, until it is proved that it is better than a basophile race, the most reasonable course to pursue is to have a universal "nitragin" for all leguminous crops, consisting of a mixed culture of active slime-forming nodule bacteria.